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MEMORIALS TO FREDERICK S. HAMMETT

Presented at the Annual Meeting
of the
Society for the Study of Development and Growth
June 20, 1953

In the passing of Frederick Simonds Hammett on April 7, 1953, a creative thinker and worker is lost to science. The Staff of the Lankenau Hospital Research Institute and the Institute for Cancer Research particularly miss him and pay tribute to the very rich part he played in the original organization of the Lankenau Hospital Research Institute.

Back in the early 1920's, he realized the importance of a more fundamental approach to the cancer problem than had been the usual practice. So when he joined the Lankenau Hospital Research Institute in September 1927 his plans and program for cancer research had been mapped. In brief, he held that cancer is but one phase of the larger problems of growth and development and should be investigated as such. This idea, it is probably fair to state, underlies most of the basic cancer research conducted at the present time. His work and his guidance and supervision in the early days of the Institute set a course which is still being pursued. His insistence on dividing growth processes into separate phases for experimental attack but continuously remembering that syntheses are necessary are now accepted methods. Always interested in the chemical aspects and thoroughly grounded in the details of biology and of chemistry, he was able to effect a happy union of the two. When his health failed around 1932, another dream of his had come true, namely, the establishment of the Marine Experimental Station of the Lankenau Hospital Research Institute at North Truro, Mass., over which he presided for 16 years. Many contributions came from this station.

He was the founder both of the Journal, GROWTH, and the Society for the Study of Development and Growth. For numerous reasons he believed that a journal specifically dealing with growth and development would be useful. In the summer of 1935 he invited a group representing various disciplines to meet in North Truro. After a few days' discussion, the journal GROWTH was launched on the principle that costs of publication should be charged to research funds, since they are as much a part of a research project as is the purchase of

test tubes. This principle has proved sound and in part has been copied by other journals.

Finding that many meetings were devoted to short papers covering many fields, his basic idea in founding the Society for the Study of Development and Growth was to pick out a topic of wide implication and have it discussed by workers in various disciplines at an annual symposium. The Society has prospered and this remains one of its basic principles.

Dr. Hammett's scientific career, before becoming attached to the Lankenau Hospital Research Institute, extended over a number of years and in a number of places. His publications attest his wide interest in problems of the chemical control of growth hormones and similar processes. While he could write lucidly when he wished, nevertheless he often produced manuscripts which exasperated his confreres. His booklet "The Nature of Growth" written without punctuation and without capital letters was one of these conceits. It repays reading.

He was a hard taskmaster, continually preaching to his associates and assistants on the necessity of employing only chemicals of utmost purity; on the use of many more control than experimental animals; on rigid cleanliness of laboratories and apparatus. He generalized rapidly; he loved arguments and often went out of his way to stir up controversy. Sometimes he was vitriolic and as can be imagined, some with whom he disputed took a long time to forget him when he showed that they were wrong.

The Staff of the Lankenau Hospital Research Institute and Institute for Cancer Research miss him even though his illness for the past three years, during which time he lived exclusively in Provincetown, incapacitated him more and more and letters and consultations with him became less and less. He maintained his interest practically to the last.

The editors of GROWTH also will feel his loss.

—STANLEY P. REIMANN

Dr. Frederick S. Hammett died early this Spring at his home in Provincetown.

Although it is not the intention of the Growth Society and the Symposium to devote to memorials to the dead many hours of time belonging to the living, we have felt that in Fred Hammett's case we should make an exception.

It has been a long time since Fred has been an active participant in our scientific community. His illness, his choice of the very unscientific surroundings of Provincetown as a retreat, many other factors have made tenuous his evident bonds to his former colleagues. To many of the younger members of this group he is only a name in the literature. But to those of us who worked with him at an earlier period he is still a very real and vibrant memory. And *we* know that without him there would have been no Symposia or Society, or Journal. I think that of that original group I am today the only member who is still taking an active part in our affairs. So it has seemed only proper that I should say a few words about him.

I first met Fred Hammett in 1935. He was already a very sick man, with progressive tuberculosis of both lungs. But he was a valiant, alert, intense, imaginative personality. He had come to recognize a not-yet-expressed but underlying need to find some means of cutting across the vertical lines of the older methodological disciplines and to consider the problems of GROWTH as a unit problem, not as a loose package of techniques. Fred invited me to Provincetown to discuss this need. Skeptical at first, I came away convinced. Out of that meeting and Fred's herculean efforts of the next two years came the Journal. That journal, in spite of its then unorthodox publication policies, financial stresses, several changes of editorship and the counter current of almost universal doubt, has survived and is this year publishing its 17th volume. It still exists because, as Fred saw, it filled a real need.

We had discussed at our earliest meetings the need for a Society to bring together those who shared this horizontal rather than vertical point of view. While recognizing that it would eventually be needed we agreed that it must evolve naturally. But Fred, from his home and laboratory on Cape Cod, wanted to help that evolution along. At his suggestion a committee, gathered from among the Editorial Board of the Journal, met in New York in November, 1938, to discuss the best means by which this might be accomplished. That committee consisted of John Berrill, Leigh Hoadley, Charles Davenport and myself, with

Stanley Reimann and Lawrence K. Frank as advisors and financial "angels." We decided that we could best promote a personal interplay by organizing a Symposium to be held at North Truro, on Cape Cod. We chose to build the first Symposium around Dr. Warren Lewis as President and Professor Joseph Needham as principal foreign guest. We arranged that a Society should be organized at the Symposium with Dr. Paul Weiss as organizing Secretary.

Through all this Fred Hammett remained in the background leaving the final organization to us. He made the physical arrangements for the Symposium which some of us remember—the School, the Whitman House cabins, the Flagship Dinner. The Symposium, which as Larry Frank remarked, represented the "C.I.O. of Science," was a great success. This year we have met for the twelfth time, our annual meetings interrupted only by two of the war years and by the joint meeting in 1950 with the International Congress for Cell Research at New Haven. The Symposia are among the outstanding biological events of the year.

This was Fred Hammett's dream. The Journal "Growth," the annual "Growth Symposium," the "Society for the Study of Development and Growth," most of what is good in the research program of the Lankenau Hospital Research Institute in Philadelphia, all of these are the creations of Fred Hammett—frail, ill, sometimes difficult but always full of worthwhile ideas and the force to carry them to fruition; in spite of his frailties a very human person and with many of the qualities of a great scientist.

—PHILIP R. WHITE
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Growth, 1953, **17**, 81-86.

STUDIES ON THE BIOTIN REQUIREMENT OF THE RAT[1]

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(Received for publication January 23, 1953)

In a series of investigations designed to explore possible relationships of biotin to the endocrine and hematopoietic systems in rats, two approaches for inducing a biotin deficiency were employed. One entailed the utilization of a diet lacking biotin and the other was the employment of a diet rich in spray-dried-egg white. After 100 days on the deficient ration none of the characteristic symptoms was observed. In those animals which received a diet fortified with egg white, the unmistakable symptomatology was noticeable as early as the 36th day following initiation to this regimen.

In view of the fact that a program of some scope was in progress, and since a biotin-free ration would obviate dietary imbalance involving excess protein intake, it was deemed important to attempt an explanation of the failure of the deficient diet to induce the desired response. For this purpose microbiological assays of samples of all rations presented to the animals were undertaken. This paper, therefore, reports on the comparative efficacy of two methods for inducing a biotin deficiency in rats and on the assay results which bear upon the diets employed.

PROCEDURE

Rats. Forty-eight weaning rats of a highly inbred strain (Wistar lineage) were divided into 6 series (8 animals per group) as follows: rats in groups I and II received a standard Purina laboratory chow (L.C.) diet; in group II as in groups III to VI inclusive, the rations were mixed thoroughly with 0.5% sulfaguanidine; group III consisted of rats presented with a biotin deficient diet (S-7 formula of Welch and Wright prepared by General Biochemicals, Inc.); the rats in group IV also received the deficient diet and in addition were injected subcutaneously with 15 μg. of biotin per day; the animals in

[1] Contribution #843 of the Massachusetts Agricultural Experiment Station.

groups V and VI were fed a diet prepared by thoroughly mixing spray-dried egg white with the finely divided standard ration in the ratio 60:40; in addition the rats in the 6th group were injected subcutaneously with 15 $\mu g.$/day of biotin. Animal weights and the quantities of food ingested were recorded for each animal twice a week.

On the 58th day after egg white feeding the deficiency symptoms were so pronounced that the rats in group V were sacrificed. At this time the group VI animals were also terminated. When after approximately 100 days no symptoms were observed in rats in groups III and IV, these plus groups I and II were sacrificed.

Microbiological Assays. The method for the determination of biotin was that proposed by Wright and Skeggs (1944) using *Lactobacillus arabinosus* 17-5 as the test organism.

Observations and Results

Rats. Figure 1 summarizes the weight data obtained from each of the 6 groups of rats. It is most apparent that the rats which received the egg white–chow mixture without supplementary biotin (group V) possess weights which are decidedly lower than those of any other group. The weight curve for these animals appears to plateau at a time coincidental with the initial observations of the incipient derangement for on the 36th day ruffled fur, alopecia, spectacle eye, and "leer mouth" were observed in most of these rats. The term "leer mouth" refers to a contracted state of the scarlet orbicularis oris muscles resulting in a pronounced and sustained baring of the central incisors. The weights of the animals on the egg white mixture but which also received biotin injections (VI) are not only superior to those of group V but also none of the characteristic signs were observable. The 2 groups of rats fed the deficient diet (III and IV) were considerably heavier than the egg white–treated animals, but there is no significant difference between the weight curves of groups III and IV even though biotin was administered to the latter animals. Also none of the animals comprising these 2 groups exhibited any symptoms of the deficiency syndrome. The highest weights were recorded for those rats which were offered the standard laboratory chow-sulfa preparation (II) for their weights were superior to those fed the unadulterated stock diet (I).

Assays. Table 1 indicates the results of the analyses for the biotin content of the test foods (standard laboratory chow (L.C.), biotin-

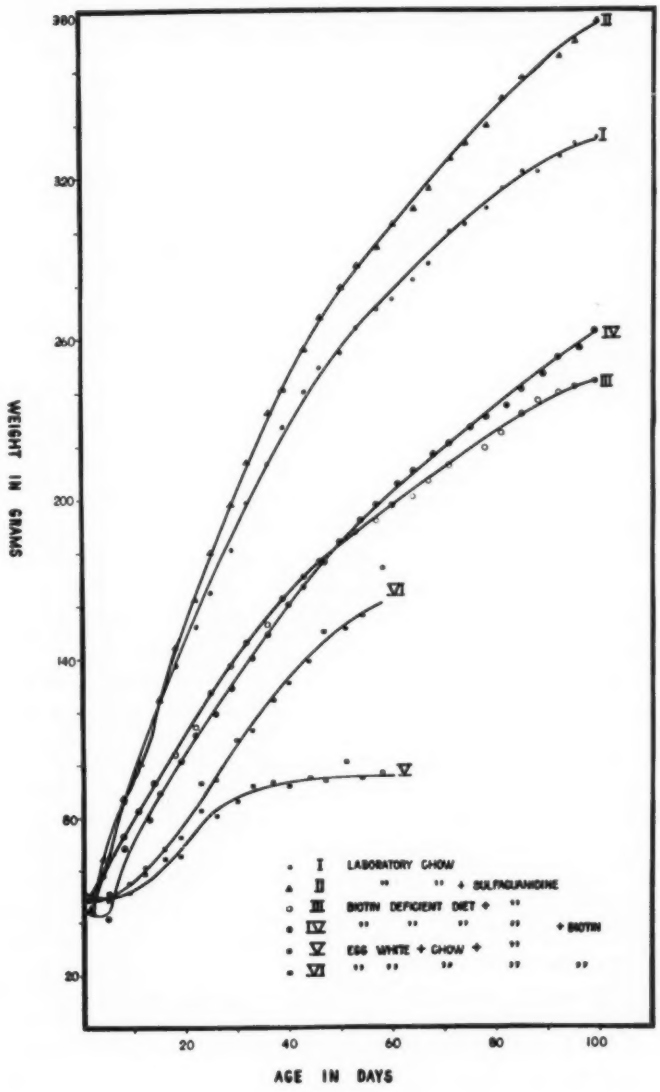

FIGURE 1
WEIGHT DATA

TABLE 1
THE BIOTIN CONTENT OF VARIOUS DIETS

Sample	Number of determinations	Average amount of biotin in *m*μg./gm.
Laboratory chow (L.C.)	3	206.00
Biotin-deficient diet (S-7)	4	00.66
Egg white–chow diet (E.C.)	3	381.00
Spray-dried egg white alone (H.L.)	3	688.00

deficient diet (S-7), egg white–chow mixture (E.C.), and egg white alone (H.L.).

By comparison with the other diets, the amount of biotin found in the deficient rations (S-7) is strikingly minute, 0.66 *m*μ*g.*/gm. It is also of interest to note that the biotin content of the egg white–chow food (E.C.) is considerably higher than that of the chow alone (L.C.). Cognizance was taken of this result and further work demonstrated that the spray-dried egg white (H.L.) contained an average of 688 *m*μ*g.* of biotin per gm. Additional analyses, utilizing samples of egg albumen obtained from another source, gave values of the same order of magnitude.

DISCUSSION

Although the deficient diet is exceedingly low in biotin, an average of 10 grams of food ingested in a 24-hour interval would provide approximately 7 *m*μ*g.* of this vitamin. Apparently this amount plus that synthesized by the intestinal flora is sufficient to have prevented the onset of the syndrome in the rats receiving this diet. Nielsen et al. (1942) reported that rats fed a purified diet containing 3 *m*μ*g.* of biotin per 10 grams of food and 0.5% sulfaguanidine developed deficiency symptoms between 3 and 5 weeks after beginning the experiments. The amount of vitamin recoverable from the feces of such animals averaged 96 *m*μ*g.*/day. If the extent of interference with the endogenous production of biotin can be assumed to be of the same order of magnitude as that reported by Nielsen, then it is tempting to conclude that the present studies approximate the minimum daily requirement of the rat for biotin more closely than has been reported heretofore. In other words, assuming the bacterial synthesis to be depressed to the same levels (since both studies employed identical concentrations of sulfaguanidine) the fact that with 3 *m*μ*g.* biotin/10 gms. of food (Nielsen) a deficiency was obtained, but with approximately 7 *m*μ*g.* under the

same conditions no deficiency resulted, would seem to indicate that the minimum value lies somewhere between these points.

The finding that considerably larger amounts of biotin are available in the egg white–chow mixture (E.C.) than in the stock diet (L.C.) alone and that this elevation is due to the egg white component receives corroboration from the work of Lampen et al. (1942) who extracted 530 *m*μ*g*. of biotin per gram of egg white using *Clostridium butylicum*. Our value (688) may be another indication of the greater precision obtainable with *L. arabinosus*. Nevertheless, despite the elevated amount of the vitamin, the rats on this diet became deficient rapidly. It is apparent, therefore, that the more efficient, even though for some purposes not the more desirable, of the two methods for inducing a biotin deficiency is the one which takes advantage of the avidin-biotin complex, namely the egg white method.

Several observations concerning the weight data merit discussion. The fact that 15 μg. of biotin/day injected into the egg white–treated animals (VI) failed to bring the weights of these animals to the level of the controls may mean that this amount falls short of the curative value in the initial stages when the weight curve parallels that for the rats receiving egg white without therapy (V).

The finding that there is little difference between the growth curves for the two groups receiving the deficient diet (III and IV) despite biotin administration to one of these (IV) would seem to indicate that there was sufficient biotin in the deficient diet (III) and that the subcutaneous addition of biotin (IV) was in excess to the rats' needs.

The observation that a group of rats on a stock diet prepared with 0.5% sulfaguanidine (group II) possess a weight advantage over group I rats receiving only the stock diet is not surprising. The literature reveals that antibiotics have been employed to favor the weight gains of fowl (Bey et al., 1950) and swine (Jukes et al., 1950). Thus it may be proper to infer that sulfa drugs possess the same ability in the rat.

Summary

Rats fed a biotin deficient diet supplemented with sulfaguanidine failed to develop deficiency symptoms during 100 days of feeding. Rats receiving a ration rich in spray-dried egg white exhibited the characteristic symptomatology beginning with the 36th day. This is indicative of the efficacy of egg white preparations in promoting a biotin deficiency syndrome in a short period of time even though large

quantities (380 *m*μg./gm.) of biotin were shown by microbiological assay (utilizing *L. arabinosus*) to be available in such a diet.

Analysis of a low biotin content diet revealed that exceedingly small amounts of biotin (of the order of 7 *m*μg./10 grams of food) were present. However, rats fed this diet plus sulfaguanidine failed to develop the deficiency. The implication that a minimum requirement range has been approximated is discussed.

Certain aspects of the growth curves of rats fed the various diets with and without biotin therapy and sulfa treatment are also discussed.

References

Berg, L. R., Bearse, C. E., & McGinnis, J. 1950. The effect of removing supplemental aureomycin from the ration on the subsequent growth of chicks. *Arch. Biochem.*, **29**, 404.

Jukes, T. H., Stokstad, E. L., Taylor, R. R., Cunha, T. J., Edwards, H. M., & Meadows, G. B. 1950. Growth-promoting effect of aureomycin on pigs. *Arch. Biochem.*, **26**, 324.

Lampen, J. O., Bahler, G. P., & Peterson, W. H. 1942. The occurrence of free and bound biotin. *J. Nutrition*, **23**, 11.

Nielsen, E., Shull, G. M., & Peterson, W. H. 1942. Response of bacteria, yeast and rats to peroxide-treated biotin. Intestinal synthesis of biotin in the rat. *J. Nutrition*, **24**, 523.

Wright, L. D., & Skeggs, H. R. 1944. Determinations of biotin with *Lactobacillus arabinosus*. *Proc. Soc. Exp. Biol. Med.*, **56**, 95.

Growth, 1953, **17**, 87-109.

THE EFFECT OF TIME OF INJECTION AND OF DOSAGE ON ABSOLUTE AND RELATIVE LENGTH OF FEMUR, TIBIOTARSUS AND TARSOMETATARSUS IN CHICKEN EMBRYOS TREATED WITH INSULIN OR PILOCARPINE[1]

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(Received for publication January 29, 1953)

Micromelia or disproportionate shortness of the extremities occurs in chicken embryos in the presence of several independent mutations as well as in consequence of a number of different dietary deficiencies. It can also be produced experimentally by treating embryos in appropriate stages with one or another of a variety of chemical compounds. In all these conditions the posterior extremities are much more involved than the anterior ones. The abnormalities of the extremities are frequently accompanied by defects of the visceral skeleton, but these latter abnormalities will not be considered in the present report.

Detailed observations on experimental micromelia of chicken embryos have been reported as a consequence of treatment with eserine and sulfanilamide (Ancel and Lallemand 1942; Ancel 1945, 1950; Zwilling and DeBell 1950), insulin, boric acid and pilocarpine hydrochloride (Landauer 1947a, 1952, 1953), and thallium salts (Karnofsky, Ridgway and Patterson 1950). Little is yet known, however, about the quantitative aspects of the experimental production of micromelia. Hence it seemed of particular interest to study the consequences to which micromelia-producing substances lead in different stages of embryonic development and to compare the effects of varying doses of a given teratogenic compound after its use at one and the same stage. Some substances, e.g. boric acid, do not lend themselves readily to such observations because they are effective only during a relatively short span of development and over a limited dosage range. For our

[1]Supported by a grant from the American Cancer Society, on recommendation by the Committee on Growth, National Research Council.

present investigations we chose insulin and pilocarpine hydrochloride because the effects of these two compounds are in some respects quite different from each other.

Material and Methods

We used eggs of White Leghorn fowl. All experimental groups and the controls were incubated simultaneously and in the same incubator. There were eight groups, one of which remained untreated. In three groups the eggs received, at 96 hours of incubation, injections of 3 mg, 6 mg and 12 mg, respectively of pilocarpine hydrochloride. The four remaining groups served for experiments with insulin. In three of these groups 2 units of insulin were injected at 84, 120 and 168 hours, respectively, the fourth being treated with 4 units at 120 hours. Injection was done into the yolk sac. The volume of fluid injected was always .05 cc., except in the case of 4 units insulin where it was .1 cc. The eggs were candled prior to injection and all infertile eggs and those with dead embryos were eliminated. The records of incubation are shown in table 1. At the end of 18 days of incubation all eggs were opened and the living embryos preserved in a solution of 5% formaldehyde in 70% alcohol. The fixing solution was changed several times. The formaldehyde-alcohol mixture was chosen to avoid excessive shrinkage or swelling.

About four months later the embryos were removed from the preserving fluid; excess fluid was allowed to drain off; the specimens were

TABLE 1

Records of control and experimental groups, showing post-injection mortality and *gross* abnormalities in percent of embryos living at 18 days

	Control	Insulin 2 U 84 hrs.	Insulin 2 U 120 hrs.	Insulin 2 U 168 hrs.	Insulin 4 U 120 hrs.	Pilocarpine 3 mg	Pilocarpine 6 mg	Pilocarpine 12 mg
Fertile eggs (treated)	96	150	147	140	150	155	164	215
Mortality to 18 days %	18.7	47.5	46.2	40.7	56.0	20.6	28.0	60.0
Fixed for measurements	75[1]	65	79	80	64	123	118	86
"Normal" %	100	61.5	31.7	27.7	27.3	83.7	49.6	15.5
Complex beak defects	—	10.8	—	—	—	—	6.5	14.5
Simple beak defects	—	3.0	11.0	12.0	22.7	2.4	4.8	27.3
Micromelia	—	33.8	67.1	69.9	72.7	1.6	—	37.9
Tibiotarsus bent	—	1.5	1.3	42.5	12.5	—	1.6	19.8
Tarsometatarsus short	—	—	—	—	—	—	22.0	35.0
and bent	—	—	1.3	—	—	—	2.4	10.7[2]
Syndactylism	—	27.7	3.7	—	3.0	14.7	49.6	78.6
Other malformations	—	—	—	2.4	1.5	1.6	1.6	1.0

[1] 3 rumpless embryos excluded; [2] also 74.8% with short first toes.

then blotted dry and weighed. Next, femur, tibiotarsus and tarsometatarsus of the left leg were dissected out and their lengths measured. This was done according to Saller's (1928) instructions for greatest length of each of the bones. Whenever bones (tibiotarsus, tarsometatarsus) were bent, the two shanks of the bent bone were measured separately and the sum was recorded.

The data thus obtained were subjected to the usual statistical analysis. Standard errors were used throughout. Beyond determining the means of body weight and bone lengths for the control embryos and the various experimental groups, our main interest lay in determining what, if any, changes in skeletal proportions had occurred as a consequence of the experimental treatments. It was obvious, however, that in the experimental groups some embryos had, with reference to length of the long bones, given no response whatever to treatment and that in others the changes were too slight to be with any certainty considered as abnormal. The percentages of such slightly affected or altogether unaffected embryos varied with the type of treatment, as will be discussed hereafter. In some of the experimental groups this lack of response was so marked that any changes in proportions among the more abnormal specimens would have been submerged in calculations for the groups as a whole.

The most extreme case of this kind is represented by the group treated with 3 mg pilocarpine. The frequency distributions of femur, tibiotarsus and tarsometatarsus length and of body weight in this group are shown in figures 1 to 4. It can readily be seen that the distributions of the data for normal and treated embryos follow a very similar pattern. A great majority of the embryos in the treated group obviously showed little or no change in length of the three long bones, the evidence for heterogeneity of distribution being most suggestive in the case of the tarsometatarsus. These conclusions are confirmed by the impressive degree of association obtaining between control and experimental groups as determined from contingency tables. The χ^2 values and probabilities were as follows: body weight $\chi^2 = 3.240$, df $= 5$, $P = .68$; femur $\chi^2 = 3.584$, df $= 4$, $P = .50$; tibiotarsus $\chi^2 = 4.606$, df $= 6$, $P = .60$; tarsometatarsus $\chi^2 = 8.034$, df $= 6$, $P = .25$; combined length of the three bones $\chi^2 = 5.069$, df $= 8$, $P = .75$.

In view of these facts, it was decided to limit our analysis of changes in *relative* length of the three long bones to embryos which showed unequivocal evidence of reduction in leg length. In order to treat all

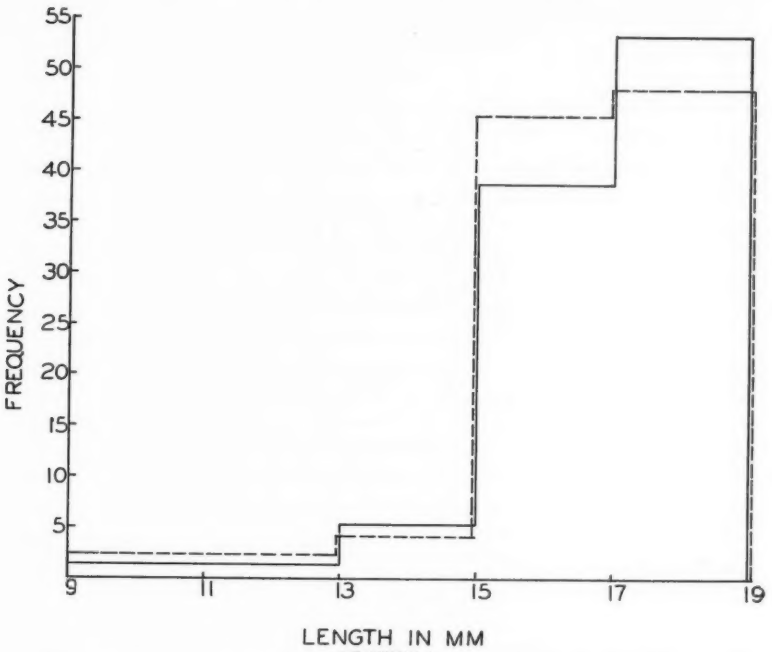

FIGURE 1

Frequency distributions of greatest femur length of 18-day chicken embryos. Solid line: untreated; broken line: 3 mg pilocarpine hydrochloride at 96 hours of incubation.

groups as uniformly as possible those embryos were excluded from subsquent analysis of the experimental groups in which combined length of femur, tibiotarsus and tarsometatarsus was equal to or exceeded the mean less 2 standard deviations for combined length of the control group (viz., $59.2 - 5.3 = 53.9$ mm). It must, therefore, be kept in mind that all conclusions concerning skeletal proportions are exclusively based on those embryos which, as a consequence of treatment with pilocarpine hydrochloride or insulin, had become definitely abnormal. They will be referred to as "grossly" abnormal embryos.

Results

Pilocarpine hydrochloride. We had previously found that the teratogenic action of pilocarpine, as far as the extremities are concerned, is limited to a short period of embryonic development. It was, therefore,

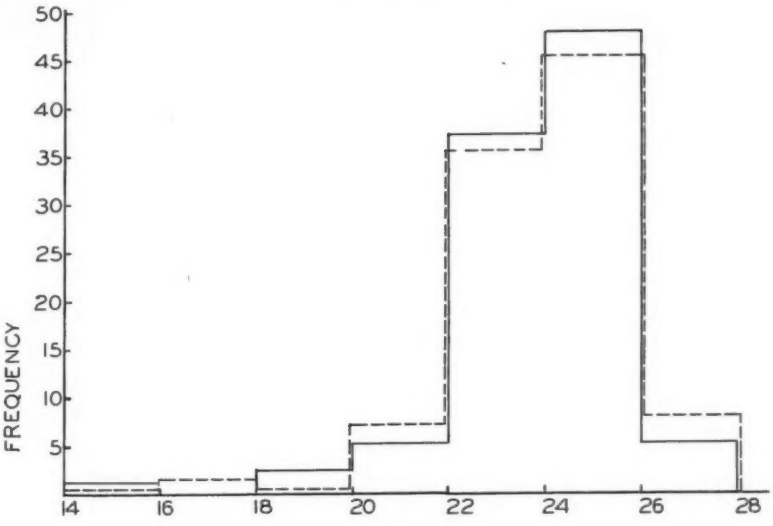

FIGURE 2

Frequency distributions of greatest tibiotarsus length of 18-day chicken embryos. Solid line: untreated; broken line: 3 mg pilocarpine hydrochloride at 96 hours of incubation.

decided to use this compound for a comparative study of the effects produced by varying amounts of pilocarpine, injected at one and the same developmental stage, viz., 96 hours of incubation. The dosages employed were 3, 6 and 12 mg, respectively. The results are shown in table 2. It can be seen that with the lowest dose, i.e. 3 mg, the effects were very slight. Among the embryos living at 18 days 14.7 percent were syndactylous (table 1), an incidence close to that reported previously. Body weight of the treated embryos did not differ significantly from the control group. Mean length of the long bones, on the other hand, was to a small but significant extent reduced in the treated embryos, with a slight trend of increasing reduction along the proximodistal axis. These statements are based on the following differences between the means of the control and treated groups:

Body weight	$0.71 \pm .396$ g
Femur length	$0.70 \pm .134$ mm
Tibiotarsus length	$1.0 \pm .22$ mm
Tarsometatarsus length	$1.1 \pm .17$ mm
Combined length of long bones	$2.9 \pm .52$ mm

TABLE 2

Body weight and length of femur, tibiotarsus and tarsometatarsus of 18-day chicken embryos after treatment with 3, 6 and 12 mg, respectively of pilocarpine hydrochloride at 96 hours of incubation and of untreated control embryos.

		Body weight g	Femur mm	Tibio-tarsus mm	Tarsometa-tarsus mm	Combined long bone length mm
Untreated	Mean	20.70 ± .297	17.4 ± .06	24.8 ± .14	18.6 ± .09	60.9 ± .31
(N = 71)	Range	8.82 — 24.88	9.8 — 18.2	15.5 — 26.5	10.3 — 19.7	35.6 — 64.4
Pilocarpine	Mean	19.99 ± .262	16.7 ± .12	23.8 ± .17	17.5 ± .14	58.0 ± .42
3 mg - 96 hrs.	Range	11.71 — 25.14	9.8 — 18.8	14.9 — 27.5	8.3 — 20.1	34.0 — 66.4
(N = 123)						
Pilocarpine	Mean	16.55 ± .348	14.9 ± .19	20.9 ± .29	14.7 ± .29	50.5 ± .76
6 mg - 96 hrs.	Range	5.43 — 23.86	6.6 — 18.0	9.1 — 25.3	4.9 — 18.5	21.8 — 61.6
(N = 118)						
Pilocarpine	Mean	11.07 ± .417	12.0 ± .09	15.7 ± .44	9.9 ± .38	37.5 ± 1.06
12 mg - 96 hrs.	Range	4.72 — 20.10	6.6 — 16.8	7.7 — 23.1	4.2 — 16.6	19.1 — 56.7
(N = 86)						

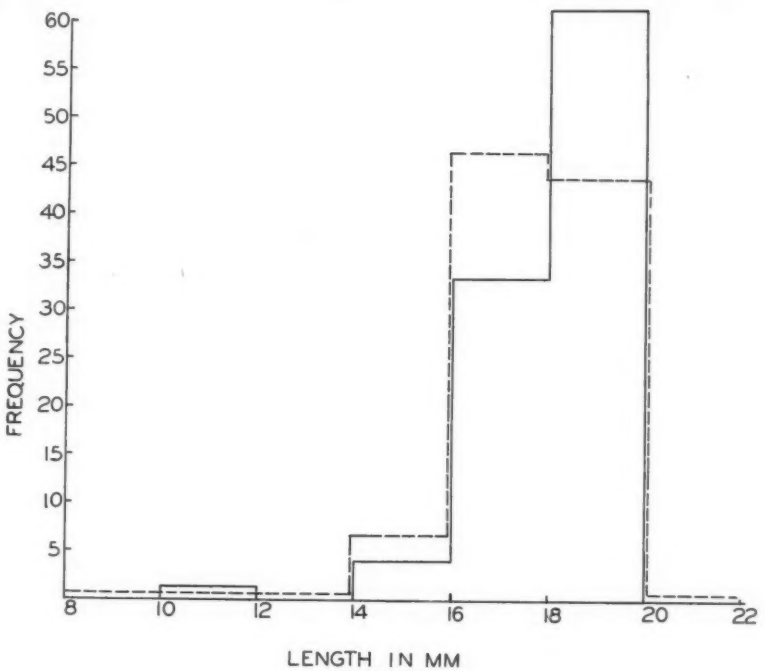

FIGURE 3

Frequency distributions of greatest tarsometatarsus length of 18-day chicken embryos. Solid line: untreated; broken line: 3 mg pilocarpine hydrochloride at 96 hours of incubation.

In the two groups which had been treated with 6 and 12 mg pilocarpine, the effects on body weight and bone length were much greater than after injection of only 3 mg. This is shown by the following figures:

	Reduction below control group	
	6 mg pilocarpine	12 mg pilocarpine
Body weight, g	4.22 ± .457	9.63 ± .512
Femur length, mm	2.5 ± .20	5.4 ± .11
Tibiotarsus length, mm	3.9 ± .32	9.1 ± .46
Tarsometatarsus length, mm	3.9 ± .30	8.7 ± .39

All these differences in weights and measurements are highly significant. Length of the femur was less affected in both groups than that of tibiotarsus and tarsometatarsus, but there was no significant differ-

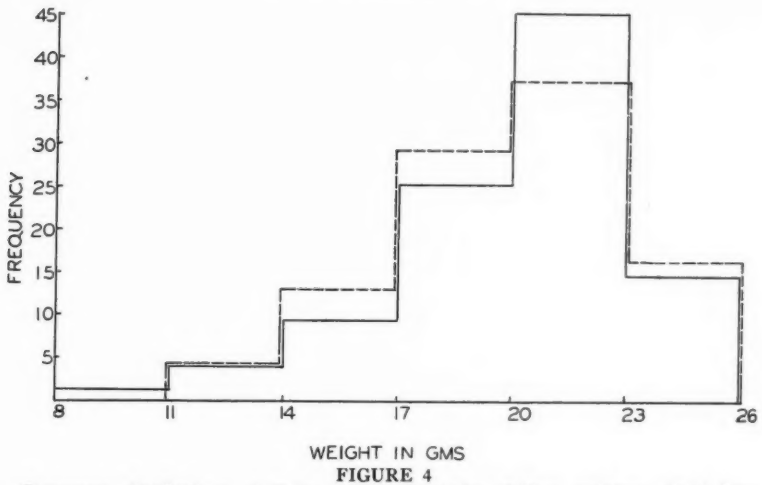

FIGURE 4

Frequency distributions of body weight of 18-day chicken embryos. Solid line: untreated; broken line: 3 mg pilocarpine hydrochloride at 96 hours of incubation.

ence in *absolute* shortening of the latter two bones. The tarsometatarsus being normally much shorter than the tibiotarsus had, of course, become relatively much the shorter of the two bones, as will be discussed more fully below.

The effect of pilocarpine treatment on mean length of the three long bones relative to their combined length and relative to the control means for each bone was studied only for those embryos for which there was indubitable evidence of shortening of the leg as a whole. The means and ranges of variation for the different measurements of these restricted groups of pilocarpine-treated embryos are reproduced in table 3. It can be seen, as might indeed be expected, that the number of embryos which reacted little to treatment or failed altogether to do so, decreased with increasing doses of pilocarpine. These differences are highly significant. The χ^2 values, calculated from 2×2 tables, were 40.99 with $P < .001$ between the 3 mg and 6 mg groups and 31.76 with $P < .001$ between the 6 mg and 12 mg groups.

Mean length of the several long bones in percent of their combined length (for those pilocarpine-treated embryos with gross reduction in combined long bone length) is shown in table 4. It is evident that, as far as growth in length is concerned, the tarsometatarsus has suffered

TABLE 3

Data for pilocarpine-treated groups after exclusion of those embryos which in combined long bone length equalled or exceeded the mean minus 2 standard deviations for combined long bone length of the control embryos.

		Body weight g	Femur mm	Tibio-tarsus mm	Tarsometa-tarsus mm	Combined long bone length mm
Pilocarpine						
3 mg – 96 hrs.	Mean	14.76 ± .49	14.1 ± .48	20.3 ± .56	14.6 ± .60	49.0 ± 1.59
(N = 15)	Range	11.71 — 17.40	9.8 — 15.5	14.9 — 22.2	8.3 — 16.4	34.0 — 53.8
Pilocarpine						
6 mg – 96 hrs.	Mean	14.47 ± .350	13.9 ± .24	19.3 ± .36	13.1 ± .35	46.4 ± .93
(N = 73)	Range	5.43 — 19.13	6.6 — 16.3	9.1 — 22.5	4.9 — 16.5	21.8 — 53.8
Pilocarpine						
12 mg – 96 hrs.	Mean	10.67 ± .387	11.8 ± .25	15.3 ± .43	9.5 ± .36	36.7 ± 1.02
(N = 82)	Range	4.72 — 18.12	6.6 — 15.6	7.7 — 21.9	4.2 — 16.1	19.1 — 52.7

disproportionately. In normal 18-day embryos the contributions by femur, tibiotarsus and tarsometatarsus to combined long bone length were 28.6, 40.7 and 30.7 percent, respectively. In the pilocarpine-treated groups of table 4 the contributions of the tarsometatarsus to combined length fell from 29.7 percent in the 3 mg-group to 28.0 percent in the 6 mg-group (difference $1.7 \pm .52$ mg) and to 25.5 percent in the group treated with 12 mg (the difference between the 6 mg and the 12 mg-groups being $2.5 \pm .47$ mg). Length of the tibiotarsus in relation to combined length remained nearly constant under the different treatments, and not very different from its proportionate length in normal embryos, whereas the femur, as the least affected of the three bones, showed a gradual increase in its share of combined length as the dose of injected pilocarpine became greater. It was found, similarly, that within a treated group the disproportions became the more emphasized the more abnormal an embryo had been rendered by treatment with pilocarpine. This is exemplified in table 5. The data for the group treated with 12 mg/egg pilocarpine are here shown in sub-groups, according to ranges of combined long bone length. It can be seen that the less was total long bone length, the smaller was the contribution of the tarsometatarsus to it. Between the lowest and highest length range there was a difference of $4.8 \pm .68$ percent in relative tarsometatarsus length. The tibiotarsus retained about the same value in the three groups, i.e. its shortening was proportional to total shortening. Femur length, on the other hand, was little involved in the individual variations in reaction to pilocarpine and as a consequence its relative length became the greater the shorter was the total combined length of the long bones.

The same conclusions emerge even more clearly when reduction in length of these long bones is expressed in percent of mean length of the same bones in the control group (table 6). It can be seen that at each dosage level the tarsometatarsus has by far most strikingly remained below the controls and that with increasing doses of pilocarpine this disproportionate reduction is rapidly becoming greater. In the two lower dosage groups, femur and tibiotarsus did not differ significantly within or between treatments. In the highest dosage group, however, there is a definite suggestion of a proximo-distal gradient of increasing retardation of bone growth in length, relative length of the tibiotarsus falling 6.0 ± 2.25 percent below that of the femur and relative length of the tarsometatarsus being 10.4 ± 2.42 percent less than that of the tibiotarsus.

TABLE 4
Mean length of femur, tibiotarsus and tarsometatarsus in percent of their combined length for pilocarpine treated embryos with gross reduction of combined long bone length.

	Femur %	Tibiotarsus %	Tarsometatarsus %
Pilocarpine 3 mg – 96 hrs. (N = 15)	28.7 ± .34	41.6 ± .34	29.7 ± .44
Pilocarpine 6 mg – 96 hrs. (N = 73)	30.2 ± .17	41.8 ± .18	28.0 ± .28
Pilocarpine 12 mg – 96 hrs. (N = 82)	32.7 ± .29	41.8 ± .21	25.5 ± .38

TABLE 5
Mean length of femur, tibiotarsus and tarsometatarsus in percent of combined long bone length. Data for embryos treated with 12 mg pilocarpine at 96 hours and showing gross defects. The data are subdivided according to three ranges in combined length of the long bones.

	Range of combined long bone length in mm		
	19–31	31.1–42	42.1–53
Femur %	35.1 ± .36	32.7 ± .36	30.2 ± .20
Tibiotarsus %	41.4 ± .49	42.6 ± .30	41.6 ± .17
Tarsometatarsus %	23.4 ± .60	24.8 ± .61	28.2 ± .32

TABLE 6
Mean length of femur, tibiotarsus and tarsometatarsus in percent of control mean. Data for pilocarpine-treated embryos with gross reduction of combined long bone length.

	Femur %	Tibiotarsus %	Tarsometatarsus %
Pilocarpine 3 mg – 96 hrs. (N = 15)	80.9 ± 2.75	81.9 ± 2.24	78.6 ± 3.18
Pilocarpine 6 mg – 96 hrs. (N = 73)	80.2 ± 1.36	77.9 ± 1.45	70.6 ± 1.87
Pilocarpine 12 mg – 96 hrs. (N = 82)	67.7 ± 1.44	61.7 ± 1.73	51.3 ± 1.95

Insulin. Our present experiments with insulin were aimed at clarifying the influence which this hormone has on long bone length as a consequence of being injected at different developmental stages as well as following the usage of different dosage levels. There were four experimental groups. In three of these, 2 units of insulin were in-

jected after 84, 120 and 168 hours of incubation, respectively; the fourth group received 4 units of insulin per egg at 120 hours. The data for the resulting weights and measurements are reproduced in table 7.

As compared with the controls, mean body weight of the insulin-treated embryos was significantly reduced in all four instances. It is remarkable, however, that the most striking lag in body weight was found in the group treated earliest (84 hours), the difference between this and the next older group (2 units at 120 hours) being highly significant ($3.44 \pm .786$ g). This is the more noteworthy as the long bones did not react in a similar fashion. Mean length of the three long bones was in all four insulin-treated-groups significantly below the control means, but within each one of the three long bones the different treatments had rather similar effects.

The incidence of gross abnormalities in long bone length (i.e., of those whose combined long bone length fell below the control mean less 2 standard deviations) varied in the insulin-treated groups with the type of treatment, as had earlier been found in the experiments with pilocarpine. Following the injection of 2 units insulin after 84, 120 and 168 hours of incubation, respectively, the incidence of embryos with grossly abnormal long bones rose successively from 62.6 to 72.2 and 77.5 percent (χ^2 between first and last group 3.823, df $= 1$, $P = .05$). For treatment with 2 and 4 units insulin at 120 hours the incidence figures were 72.2 and 85.8 percent ($\chi^2 = 6.0$, $P < .02$). Table 8 gives the means of weights and measurements after exclusion of the embryos without gross abnormality of combined long bone length.

The proportions of the different long bones to each other showed surprisingly small changes (table 9). The tibiotarsus was relatively shorter than normal in all treated groups and the extent of this disproportionate shortening seemed to become the more marked the later the insulin had been injected. These differences are slight, but undoubtedly real as the further analysis will show. A similar situation was found when mean length of the long bones was expressed in percent of the control means. The tibiotarsus was again consistently shorter than femur and tarsometatarsus (table 10). In order to determine if relative length of the tibiotarsus varied with the extent to which long bone length had become abnormal as a consequence of insulin treatment, the data for the groups treated with 2 units of insulin were

TABLE 7

Body weight and length of femur, tibiotarsus and tarsometatarsus of 18-day chicken embryos after treatment with 2 units insulin at 84, 120 and 168 hours of incubation, with 4 units insulin at 120 hours and of untreated controls.

		Body weight g	Femur mm	Tibio-tarsus mm	Tarsometa-tarsus mm	Combined long bone length mm
Untreated	Mean	20.70 ± .297	17.4 ± .06	24.8 ± .14	18.6 ± .09	60.9 ± .31
(N = 71)	Range	8.82 — 24.88	9.8 — 18.2	15.5 — 26.5	10.3 — 19.7	35.6 — 64.4
Insulin						
2 U – 84 hrs.	Mean	15.45 ± .639	13.6 ± .36	19.1 ± .59	14.6 ± .44	47.3 ± 1.38
(N = 64)	Range	4.95 — 23.39	5.9 — 17.5	6.5 — 24.4	4.2 — 18.9	17.4 — 60.4
Insulin						
2 U – 120 hrs.	Mean	18.89 ± .458	13.6 ± .29	18.9 ± .45	15.3 ± .31	47.8 ± 1.04
(N = 79)	Range	7.96 — 24.44	6.1 — 17.8	8.4 — 26.0	7.2 — 19.8	22.7 — 63.3
Insulin						
2 U – 168 hrs.	Mean	17.38 ± .574	13.3 ± .78	17.6 ± .61	14.2 ± .44	45.1 ± 1.43
(N = 80)	Range	6.74 — 25.17	6.6 — 18.2	7.6 — 25.7	6.6 — 19.8	21.8 — 63.2
Insulin						
4 U – 120 hrs.	Mean	16.86 ± .513	12.6 ± .33	17.1 ± .51	13.9 ± .42	43.7 ± 1.24
(N = 64)	Range	6.47 — 23.75	6.7 — 17.5	8.2 — 24.7	5.8 — 18.8	20.7 — 61.0

TABLE 8

Means of body weight and length of long bones for insulin-treated groups of table 6 after exclusion of embryos which did not show gross abnormality of combined long bone length.

		Body weight g	Femur mm	Tibio-tarsus mm	Tarsometa-tarsus mm	Combined long bone length mm
Insulin						
2 U – 84 hrs.	Mean	13.17 ± .641	12.1 ± .42	16.7 ± .72	12.9 ± .54	41.7 ± 1.65
(N = 40)	Range	4.95 — 19.88	5.9 — 15.6	6.5 — 21.5	4.2 — 16.6	17.4 — 53.4
Insulin						
2 U – 120 hrs.	Mean	17.18 ± .511	12.4 ± .29	16.9 ± .44	13.9 ± .31	43.2 ± 1.02
(N = 54)	Range	7.96 — 23.33	6.1 — 15.9	8.4 — 22.2	7.2 — 16.7	22.7 — 53.8
Insulin						
2 U – 168 hrs.	Mean	16.14 ± .460	12.4 ± .31	15.9 ± .43	13.1 ± .33	41.4 ± 1.05
(N = 62)	Range	6.74 — 22.30	6.6 — 16.2	7.6 — 21.0	6.6 — 17.3	21.8 — 53.7
Insulin						
4 U – 120 hrs.	Mean	16.15 ± .531	12.1 ± .32	16.2 ± .49	13.2 ± .41	41.5 ± 1.21
(N = 55)	Range	6.47 — 22.04	6.7 — 15.5	8.2 — 21.0	5.8 — 17.3	20.7 — 53.7

subdivided according to combined length of the long bones, and for each sub-group mean tibiotarsus length was determined in percent of combined length. The results show (table 11) that the more combined length of the long bones fell below normal, the less did the tibiotarsus contribute to total length of the long bone axis. This trend is slight, but consistent. Neither femur nor tarsometatarsus gave indications of a definite trend in relative length.

TABLE 9

Mean length of femur, tibiotarsus and tarsometatarsus in percent of their combined length for insulin-treated embryos with gross reduction of combined long bone length and for untreated control embryos.

	Femur %	Tibiotarsus %	Tarsometatarsus %
Untreated (N = 71)	28.6 ± .066	40.7 ± .074	30.7 ± .059
Insulin 2 U – 84 hrs. (N = 40)	29.3 ± .319	39.9 ± .406	30.8 ± .425
Insulin 2 U – 120 hrs. (N = 54)	28.7 ± .155	39.0 ± .210	32.3 ± .224
Insulin 2 U – 168 hrs. (N = 62)	29.8 ± .243	38.4 ± .228	31.6 ± .235
Insulin 4 U – 120 hrs. (N = 55)	29.3 ± .176	39.2 ± .195	31.7 ± .211

TABLE 10

Mean length of femur, tibiotarsus and tarsometatarsus in percent of control mean. Data for insulin-treated embryos with gross reduction of combined long bone length.

	Body weight %	Femur %	Tibiotarsus %	Tarsometatarsus %
Insulin 2 U – 84 hrs. (N = 40)	63.6 ± 3.09	69.1 ± 2.46	67.5 ± 2.89	69.3 ± 2.89
Insulin 2 U – 120 hrs. (N = 54)	83.0 ± 2.47	71.2 ± 1.67	68.3 ± 1.79	74.7 ± 1.68
Insulin 2 U – 168 hrs. (N = 62)	78.0 ± 2.22	71.2 ± 1.76	64.2 ± 1.75	70.2 ± 1.79
Insulin 4 U – 120 hrs. (N = 55)	78.0 ± 2.57	69.3 ± 1.85	65.5 ± 1.98	70.8 ± 2.21

TABLE 11

Mean length of tibiotarsus in percent of combined long bone length, in groups according to ranges of combined long bone length. Data for those embryos treated with 2 units of insulin in which combined long bone length was grossly abnormal.

Insulin 2 U – 84 hrs.	Range of combined length mm	17.4 — 29.0	29.1 — 41.0	41.1 — 53.4
	Mean relative tibiotarsus length %	37.7 ± 1.92	39.8.± .32	40.6 ± .22
Insulin 2 U – 120 hrs.	Range of combined length mm	22.7 — 33.2	33.3 — 43.7	43.8 — 53.8
	Mean relative tibiotarsus length %	38.2 ± .95	38.4 ± .44	39.6 ± .19
Insulin 2 U – 168 hrs.	Range of combined length mm	21.8 — 32.0	32.1 — 43.0	43.1 — 53.7
	Mean relative tibiotarsus length %	37.4 ± 1.09	38.3 ± .21	38.8 ± .58

It has been seen that treatment with insulin or pilocarpine, at the given developmental stages, tends to lead to reduced body weight as well as to subnormal length of one or more of the three long bones of the leg. Our data have shown quite clearly that within the long bone axis pilocarpine has a differential effect, growth in length of the tarsometatarsus being much more depressed than that of femur and tibiotarsus. Insulin treatment, in contrast, resulted in a more uniform shortening of the three long bones of the leg. It seemed, therefore, desirable in this instance to inquire into the relations between changes of body weight and of bone length. Coefficients of correlation between body weight on the one hand and length of femur, tibiotarsus and tarsometatarsus on the other were calculated for the three groups treated with 2 units insulin at 84, 120 and 168 hours of incubation, respectively, and these are reproduced in table 12. It can be seen that a high positive correlation obtains in all instances and the relative uniformity of the results is fairly impressive. This, of course, does not necessarily

imply a causal dependence of one effect upon the other, but it shows that in spite of a great range of response differences between embryos, the effects on general body size and on length of the long bones of the legs tend to maintain a fairly uniform relationship.

Gross observations show, however, that the effect of insulin on leg length is greater than that on body size, resulting in micromelia or disproportionate shortness of the legs. This is in a general way confirmed by the regression coefficients given in table 13. It can be seen that, with one exception, the coefficients for regression of body weight on bone length (bxy) are always greater than those for regression of length on weight, indicating that the disproportion of bone length became the more marked (the degree of micromelia the greater) the more extreme was the effect of insulin on body weight. The one exception to this rule was the behavior of the tibiotarsus in the group treated at 84 hours. It will be remembered that in this group body weight was much lower than in the groups treated in later stages. It will, however, be observed, in addition, that in comparison to femur and tarsometatarsus the tibiotarsus coefficients for bxy are always smaller and those for byx are always larger than is true for the two other bones. This is an expression of the considerably greater variability in length of the

TABLE 12

Coefficients of correlation between long bone length and body weight in groups treated with 2 units insulin between 84 and 168 hours of incubation and for embryos showing gross abnormality of combined long bone length.

	Coefficient of correlation between body weight and length of		
	Femur	Tibiotarsus	Tarsometatarsus
Insulin – 84 hrs.	+ .860	+ .846	+ .856
Insulin – 120 hrs.	+ .853	+ .849	+ .846
Insulin – 168 hrs.	+ .850	+ .793	+ .832

TABLE 13

Values indicating the regression of long bone length on body weight (byx) and of body weight on long bone length (bxy) in groups treated with 2 units insulin between 84 and 168 hours of incubation and for embryos showing gross abnormality of combined long bone length.

	Femur		Tibiotarsus		Tarsometatarsus	
	byx	bxy	byx	bxy	byx	bxy
Insulin 84 hours	.563	1.314	.943	.759	.727	1.007
Insulin 120 hours	.486	1.498	.738	.977	.517	1.385
Insulin 168 hours	559	1.293	.730	.861	.594	1.165

tibiotarsus than of femur and tarsometatarsus in all insulin-treated groups.

Discussion

Briefly recapitulated, the more important observations on our material were as follows: After treatment with 2 units insulin between 84 and 168 hours of incubation, the incidence of embryos with gross abnormality of the leg bones and of body weight rose with successively later stages of treatment. Such a rise in incidence was also produced by using increasing amounts of a teratogenic substance at one and the same stage (pilocarpine at 96 hours, insulin at 120 hours). Body weight was significantly lower than normal after all treatments, except that with 3 mg pilocarpine at 96 hours. At 84 hours of incubation 2 units insulin had a more adverse effect on body weight than in subsequent stages. Within one developmental stage mean length of the long bones depended on the amount of injected teratogenic compound, but 2 units insulin used in various stages (84 to 168 hours) did not produce any obvious stage differentials. Among grossly abnormal treated embryos, it was found that pilocarpine produced the least alteration in relative length (relative to combined length of the three long bones or in terms of the control mean for each bone) of the femur, the greatest in that of the tarsometatarsus, with the tibiotarsus occupying an intermediate position. The changes in relative length of the long bones were much less pronounced after insulin treatment; the principal effect on relative length, irrespective of stage and dosage, was on the tibiotarsus; there were no significant or consistent differences in the reactions shown by femur and tarsometatarsus. The greater the mean reduction of combined long bone length or the lower the body weight the more pronounced was the relative shortening of the *tibia* after insulin treatment; a similar trend of increasing relative shortness of the tibia (within the long bone axis) prevailed from early (84 hours) toward late (168 hours) stages of treatment. After pilocarpine treatment, on the other hand, length of the *tarsometatarsus* suffered the more, the greater was the reduction in combined length of the three long bones. Different dosages, whether of insulin or pilocarpine, used at one and the same stage, did not alter the sequence of involvement of the three long bones, typical for the particular teratogenic compound; increasing amounts merely served to exaggerate the disparities between the several bones.

It has been repeatedly pointed out in earlier publications and is confirmed by our present material that such teratogenic substances as

insulin, sulfanilamide, eserine, boric acid and pilocarpine produce many similarities in their over-all effects on developing chicken embryos, but that between the syndromes to which they give rise there are also many and important dissimilarities in detail of expression. The data from experiments with insulin and pilocarpine, reported here, demonstrate that both of these compounds tend to interfere with general body growth (weight) and that embryos exposed to them are likely to develop more or less extreme symptoms of micromelia and defects of the visceral skeleton. It is, furthermore, known that in combination with all the teratogenic substances that have been enumerated, nicotinamide serves as a protective supplement, thus pointing to a common biochemical basis for the activity of these compounds.

It is important to note, however, that the effectiveness of nicotinamide in forestalling malformations is not the same in all instances. In the case of insulin (2 units at 96 or 120 hours), sulfanilamide (1.6 to 1.8 mg at 48 and 120 hours) or eserine (0.25 to 0.5 mg at 96 hours) the appearance of skeletal defects could be entirely prevented with proper doses of nicotinamide. In conjunction with boric acid and pilocarpine, on the other hand, nicotinamide gave only partial protection, and this indication of a somewhat different biochemical mechanism is, at least in the case of boric acid, supported by evidence from other sources (Landauer, 1952).

Those experimentally-produced micromelias which are most readily preventable by nicotinamide, viz. the ones due to treatment with insulin, sulfanilamide or eserine, appear to be characterized by somewhat similar proportions of the affected long bones. It is evident from the data here reported for insulin and from the observations by Zwilling and DeBell on sulfanilamide-induced micromelia that in both instances the femur is least and the tibiotarsus most affected, with the tarsometatarsus holding an intermediate position. The same is probably true for micromelia due to treatment with eserine, although this conclusion is only based on gross observations, not on measurements. In contradistinction, the micromelias brought on by pilocarpine and boric acid have in common that the tarsometatarsus shows by far the greatest reduction in length.

These variations in the extent to which different segments of the long bone axis are affected by different treatments can not be explained on the basis of dissimilarities in the time elapsing between injection of the teratogens and their becoming effective. For, the present data show

very impressively that over a surprisingly long span of embryonic development, viz. 84 to 168 hours of incubation, insulin treatment led to the same sequence of shortening of the three long bones (tibiotarsus > tarsometatarsus > femur).[1]

The only features of the insulin-produced syndrome which showed a definite stage dependence within the developmental period under investigation were syndactylism and bending of the tibiotarsus, two abnormalities which are probably secondary morphogenetic effects of growth dislocations. In experiments with 2 units insulin syndactylism, i.e. a webbing of the third and fourth toe, occurred with an appreciable incidence only following injection at 84 hours of incubation (27.7%); there were a few cases (3.7%) as a consequence of treatment at 120 hours, but none after injection at 168 hours. Treatment with pilocarpine, a substance which, as has been seen, exerts within the long bone axis its principal effect on the tarsometatarsus, still yielded a high incidence of syndactylism when injected at 96 hours and these pilocarpine-induced cases of syndactylism tended to be more extreme, frequently involving partial fusion of the third and fourth toe. This dissimilarity of reaction seems at least in part to be the result of differing affinities of the teratogenic compounds, pilocarpine tending to have more distal effects than insulin; for, it can be seen that even at 96 hours and with a dose of 6 mg/egg (leading to a high incidence of syndactylism), the total shortening of the long bone axis is on the average less than that produced by 2 units insulin at any time between 84 and 168 hours of incubation.

The high incidence of bending of the tibiotarsus caused by the injection of 2 units insulin at 168 hours, an abnormality that makes its appearance prior to or at the very beginning of periosteal ossification (and that Ancel, on somewhat doubtful grounds, explained as a consequence of faulty or incomplete fusion at the tibio-tarsal junction) must, on the other hand, be ascribed to (as yet undefined) peculiarities of the tibiotarsus in this particular stage since no comparable incidence followed treatment with an even higher dose (4 units) at 120 hours.

The principal effect of our various micromelia-producing compounds is unquestionably on growth (cellular multiplication) rather than on differentiation of specific parts and tissues. It is equally clear, however, that great differences exist in local sensitivity to one compound

[1]Statements in conflict with our observations have been made by Duraiswami (1950), but need not be discussed since they were offered without documentary evidence of any kind.

or another. In experiments with insulin and pilocarpine the damaging effects on growth of body and long bones presumably occurred soon after injection, the final result representing an accumulation of compound interest. This conclusion is indicated by the fact that supplementary nicotinamide lost its protective quality unless the embryo was provided with it at or very soon after the time of treatment with insulin or pilocarpine. The situation is somewhat different with sulfanilamide as has been shown by Zwilling and DeBell. Very early injection of this compound (48 hours) produced a high incidence of micromelia which could still be forestalled by providing supplementary nicotinamide 3 days later, suggesting that the sulfanilamide remained ineffective until the long bones reached a stage sensitive to it.

The disproportionate retardation of long bone growth (micromelia) is certainly not a secondary consequence of a general retardation of body growth. This is shown by the fact that in the experience of Zwilling and DeBell the long bones are more amenable to protection with supplementary nicotinamide than is body weight. It is also attested by the fact that with different treatments the effects on body weight and long bone length may vary independently; thus 6 mg pilocarpine given at 96 hours resulted in a significantly lower body weight than that produced by 2 units insulin at 120 hours, though in the insulin group, femur and tibiotarsus were significantly shorter than in that treated with pilocarpine (and tarsometatarsus length did not differ significantly between the two groups). Also, it is known that drastic retardation of general body growth need not be accompanied by micromelia (e.g., after treatment with adrenal cortical extract, Landauer, 1947b, or with para-aminobenzoic acid, Zwilling and DeBell).

Our observations have made it clear that differences must exist between such teratogenic substances as insulin and pilocarpine in the biochemical pathways by which they produce micromelia and other abnormalities, although the fact that after all these different treatments the embryos are in varying degrees amenable to protection by supplementary nicotinamide, suggests that related reactions are responsible in all instances. It is possible, on the other hand, that each one of the teratogenic compounds produces a biochemical reaction with only one widely used cellular constituent for which the demand and the ways in which it is utilized differ greatly between parts and organs of the body, thereby leading to differential results. The dissimilarity of quan-

titative effects which teratogenic compounds may produce on body size and extremities, the disproportions which they bring about in length of the long bones, the disparity between different compounds with regard to which one of the long bones suffers most, presumably are a vivid illustration of the complexities of biochemical differentiation.

Summary

Observations have been made on body weight and on length of femur, tibiotarsus and tarsometatarsus of 18-day chicken embryos which had in earlier developmental stages been treated with insulin or pilocarpine. The more important results were as follows:

Body weight did not differ significantly from normal after treatment with 3 mg pilocarpine at 96 hours of incubation. It was below normal after 6 or 12 mg pilocarpine at 96 hours, after 2 units insulin at 84, 120 or 168 hours and after 4 units insulin at 120 hours. Two units insulin at 84 hours had a more adverse effect on body weight (but not on long bone length) than treatment with the same amount at 120 or 168 hours.

Mean length of the three long bones was less than normal subsequent to all the above treatments. When different amounts of the same teratogenic compound were used at one developmental stage, it was found that the higher dose led to more extreme shortening of the long bones. Mean length (mean extent of shortening) of each of the three long bones was about the same following treatment with 2 units insulin at 84, 120 or 168 hours of incubation.

The percentage of embryos with gross symptoms of micromelia increased with dosage, if pilocarpine or insulin were used at one developmental stage. The incidence also rose with time when a constant dose of 2 units insulin was injected between 84 and 168 hours.

After pilocarpine treatment the greatest reduction in relative length occurred in the tarsometatarsus; relative femur length was least affected. The larger the injected dose, the more extreme was this differential effect on the three long bones. Within each dosage group the differential was the more marked, the shorter was combined length of the three long bones or the lower weight of the body.

After insulin treatment the tibiotarsus showed the greatest reduction in relative length, relative femur length was least affected. Stage of development at the time of injection (between 84 and 168 hours) had no effect on this sequence of interference with growth in length of the

three bones. The later the time of injection of insulin, or the larger the amount injected, or the more severe the response of an individual embryo, the more exaggerated became the disproportionate shortening of the tibiotarsus.

Acknowledgments

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References

Ancel, P. 1945. L'achondroplasie. Sa réalisation expérimentale— sa pathogénie. *Annales d'Endocrin.*, **6**, 1-24.

———. 1950. La chimiotératogenèse. Paris (Doin).

———. & Lallemand, S. 1942. Sur une malformation du bec et des membres obtenue chez l'embryon de poulet a l'aide de sulfamides. *C. R. Soc. de Biol.*, **136**, 255-256.

Duraiswami, P. K. 1950. Insulin-induced skeletal abnormalities in developing chickens. *British Med. Jour.*, **2**, 384-390.

Karnofsky, D. A., Ridgway, L. P., & Patterson, P. A. 1950. Production of achondroplasia in the chick embryo with thallium. *Proc. Soc. Exp. Biol, and Med.*, **73**, 255-259.

Landauer, W. 1947a. Insulin-induced abnormalities of beak, extremities and eyes in chickens. *J. Exp. Zool.*, **105**, 145-172.

———. 1947b. Potentiating effects of adrenal cortical extract on insulin-induced abnormalities of chick development. *Endocrinology*, **41**, 489-493.

———. 1952. Malformations of chicken embryos produced by boric acid and the probable role of riboflavin in their origin. *J. Exp. Zool.*, **120**, 469-508.

———. 1953. On teratogenic effects of pilocarpine in chick development. *J. Exp. Zool.*, 122, 469-483.

Saller, K. 1928. Die Methodik biometrischer Messungen an Laboratoriumsversuchstieren. *Abderhalden's Handbuch der biologischen Arbeitsmethoden*, **VII (1)**, 683-840.

Zwilling, E., & DeBell, J. T. 1950. Micromelia and growth retardation as independent effects of sulfanilamide in chick embryos. *J. Exp. Zool.*, **115**, 59-81.

Growth, 1953, **17**, 111-122.

CULTIVATION AND MORPHOLOGY OF HUMAN TISSUE MAST CELLS in vitro[1]

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INTRODUCTION

Tissue mast cells are greatly increased in urticaria pigmentosa in man (17) and in "mastocytomas" in dogs (1). The history of these cells up to 1938 has been reviewed by Michels (10). A comprehensive report of the literature since that date has been published by Friberg et al. (5).

Serial biopsies from a patient with urticaria pigmentosa have been described by Drennan (2) and compared with biopsies rich in mast cells, from the normal tissues of man and the rat. Ellis (4) has undertaken a detailed histologic study of the increase of mast cells in the organs, taken at complete autopsy, from an infant that died in the active phase of the disease.

The relation between mast cells and heparin has been described by Holmgren and Wilander (8) and Jorpes (9). They found that the number of mast cells corresponded to the quantity of heparin in various organs. This has been confirmed by Oliver et al. (11) who assayed mast cell tumors from dogs, and by Ehrich et al. (3) who reported a case of elephantiasis of the scrotum with excessive numbers of mast cells.

Paff et al. (12, 13, 14) have succeeded in cultivating mast cells from "mastocytomas" of dogs. They observed that only mast cells grew in these tissue cultures, and that aging tumor mast cells liberated a metachromatic substance.

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The present study is concerned with the cultivation *in vitro,* of human tissue mast cells from a biopsy of an infant with urticaria pigmentosa. The ten month old infant was admitted to hospital two months prior to the time that a section of skin was removed from the base of the neck. The infant died of pulmonary edema one month later.

Culturing Techniques

Materials and methods in general use in this laboratory (15) were employed and modified for this experiment. Tyrode's solution, lyophilized chick plasma, and fresh chick embryonic extract, diluted 1 : 1 with Tyrode's solution (hereafter referred to as EE_{50}) were used throughout this study. Various sera were introduced to stimulate optimal growth. Antibiotics were later used to insure sterility. Three culturing techniques were followed:

(1) D3.5 Carrel flasks offered the opportunity for microscopic examination and subculturing. Six tissue fragments, placed circularly, were imbedded in 0.5 ml plasma and 1.0 ml coagulum, containing 20% EE_{50}, 40% serum, and 40% Tyrode's solution. After clotting, 1.0 ml of the culture medium was added. 5% CO_2 in air was used to adjust the pH.

(2) Roller tubes were employed to promote rapid growth. Six tissue fragments were placed in a vertical line and covered with a thin layer of clot, composed of 0.2 ml plasma and 0.1 ml EE_{50}. 2.0 ml of the culture medium were added. 5% CO_2 in air was used to adjust the pH.

(3) Hanging-drop slides were prepared to facilitate staining, phase microscopy, and photography. The tissue fragments were imbedded in one drop each of 1:1 plasma, EE_{50}, and serum. All cultures were incubated at 36°C. The media were replaced once or twice weekly as the reduction in pH indicated.

Experimental

The skin biopsy, 5 × 10 mm, was stripped of subcutaneous fat and cut into 1 mm cubes. These were repeatedly washed in fresh Tyrode's solution before implanting. Three initial media were used:

1. 40% Healthy Human Adult Serum; 20% EE_{50}; 40% Tyrode's solution.
2. 40% Horse Serum; 20% EE_{50}; 40% Tyrode's solution.
3. 50% Horse Serum; 10% EE_{50}; 40% Tyrode's solution.

Medium 1 stimulated optimal growth and was adopted for routine use. Healthy human adult serum was difficult to procure in adequate amounts and was replaced by pooled serum from ward patients with hypertension. Calcification developed after two months and destroyed many cultures. This was overcome to a high degree by reducing the concentration of embryonic extract as recommended by Hanks (7). A level of 8% EE_{50}, giving a serum-extract ratio of 10:1, was established and maintained.

The addition of antibiotics was instituted at this time also. Thenceforth each milliliter of medium contained 50 units of sodium penicillin and 50 micrograms of streptomycin.

In the hope of stimulating cytoplasmic granulation, the Tyrode's solution was altered by the addition of organic and inorganic sulfur: 100 mg. magnesium sulfate (replacing the magnesium chloride), 10 mg. glutathione, and 10 mg. cysteine hydrochloride per liter.

Since human tissue mast cells had not been cultivated previously, there were no criteria on which to base these experiments and it took four months to establish a satisfactory procedure. The following additional types of media were investigated:

Medium Number	Serum	EE_{50}	Antibiotics	Tyrode's solution (mod.)
4	40% Preadolescent (eight to nine years)	8%	5%	47%
5	40% Leukemic (six year child)	"	"	"
6	40% Cord	"	"	"
7	40% Simms SUF	"	"	"
8	40% Cord	none	"	55%
9	60% Cord	"	"	35%
10	40% Simms SUF	"	"	55%
11	60% Simms SUF	"	"	35%
12	20% Cord and 20% Simms SUF	8%	"	47%
13	40% Cord and 20% Simms SUF	"	"	27%
14	Medium 6 was withdrawn from actively growing cultures at forty-eight hours and fed as "conditioned" medium to new subcultures.			
* 15	Medium 6 was used as a base for testing Growth Hormone. The purified material was diluted with Tyrode's solution and passed through a fritted glass filter. Each ml of medium contained 24 μg Hormone.			

Since urticaria pigmentosa is primarily a disease of childhood it was hoped that serum from preadolescents might contain a factor essential to the cultivation of mast cells.

*Kindly supplied by Dr. Chaoh Li, Department of Biochemistry, University of California, Berkeley.

Cord serum and serum ultrafiltrate were used without embryonic extract in an attempt to stimulate differentiation as described by Gaillard (6).

Serum ultrafiltrate, as recommended by Simms (16), was used as a constituent of the medium with embryonic extract, to observe its effect on cytoplasmic granulation.

Cultures with heavy calcium phosphate deposits were washed in a 5% saline solution of trypsin (sterilized by filtration) for five to ten minutes. Fresh plasma and coagulum were then applied.

Results

The cultures underwent four distinct phases in the course of this study. The first two month period concerned the original cultures and subcultures with a high concentration of EE_{50} employed in the medium. The first tissue fragments showed no evidence of growth until the seventh day, when a few spindle-shaped, granulated cells were observed in one Carrel flask culture maintained on Medium 1 (human serum). At ten days this culture and one other Carrel flask culture maintained on the same medium showed a fringe of new cells emerging from each tissue fragment, and there was a marked drop in pH. At two weeks these cultures had extensive and dense growth zones containing cells with well-defined spindles, packed with cytoplasmic granules, and large, regular nuclei. The slight evidence of growth noted in the remaining Carrel flask, roller tube, and hanging-drop cultures at two weeks, failed to demonstrate further cell activity. The cells became rounded and appeared dead, and were discarded at the end of the first month.

The first subcultures were made from Carrel flasks at fifteen days. New cells, seen at forty-eight hours, grew rapidly in a dense radial network and maintained cytoplasmic granulation. A smear preparation from a subculture fragment stained metachromatically with toluidine blue. Phase microscopy revealed that the densely packed cytoplasmic granules varied greatly in size. Four successful transfers were made during the first two months of cultivation.

Roller tube and hanging-drop cultures, prepared from Carrel flask subcultures, failed to grow, although the cells remained viable, as demonstrated by subsequent replanting into Carrel flasks in which proliferation occurred. Haphazard migration of a few cells was always noted in hanging-drop cultures, and the migrating cells could be

readily observed, photographed, and stained. These culturing techniques were tried repeatedly during various phases of the experiment but were never successful.

The second period, the third and fourth months, was marked by the losses sustained through calcification and the efforts made to overcome them and establish a satisfactory procedure. At the end of the fourth month no original tissue remained. A few second and third generation cultures became the only source of experimental material. Although the clots were opaque, the cells were not destroyed or injured. Portions of these cultures, with dense cell growth, were removed and successfully transplanted into new Carrel flasks. The remaining portion of the culture was reclotted in the same flask. The regenerated cells which filled the reclotted or patched area were not regarded as a new generation, and were used again for subculturing when adequate growth had been attained. Repeated subculturing and patching in these old

FIGURE 1
Third generation living, unstained culture. × 180

cultures stimulated excellent growth. The cells became highly differentiated, with thickened spindles and dense, coarse, cytoplasmic granulation.

One second generation culture was maintained in the same flask, with weekly medium changes, for a period of four months. No clot lysis occurred during this time. The explant area measured 1 cm in diameter of which the surrounding halo measured 0.4 cm. The cells grew in a dense radial pattern composed of single-cell-wide rows. The peripheral cells were large, some of them 600 microns in length, and great pleomorphism was noted among them. No rounded forms and no free granules suggesting cell dissolution, were seen. Subcultures made at this time were successful. The addition of organic and inorganic sulfur to the Tyrode's solution showed no effect upon cytoplasmic granulation.

During the next four-month period six transfers were accomplished. Growth was uniform and abundant. Cytoplasmic granulation varied, however, and prompted the investigation of different media. The following results were obtained:

Medium 6 (Cord Serum) became the basic supernatant. It stimulated adequate but not optimal growth. It maintained the cultures and promoted proliferation for five months. Mitotic and amitotic divisions were observed. Proliferation was noted 24 to 48 hours after subculturing had been undertaken. The small, well-granulated spindles formed a dense, radial network. Long, delicate filaments with a single line of granules extended from the peripheral cells. This pattern was followed during the first week after which swirls and streamers of cells, dense bridges, and syncytial forms replaced the radial design. The peripheral cells became flattened and amoeboid in shape, there was a marked loss of granulation, and the nuclei became enlarged with two or four nucleoli. At three weeks many small black deposits were seen among the cells, possibly ruptured granules. In general, the cells were pale or contained some spotty, fine granulation with no new cells noted and no change in cell structure. The cultures responded indifferently to any new stimulus. At five weeks the flattened peripheral cells became spindled, granulation was again seen, and growth continued.

Medium 4 (Preadolescent Serum) gave the same stimulus to subcultures as that described for Medium 6. Since it was more difficult to procure in adequate amounts and showed no evidence of contain-

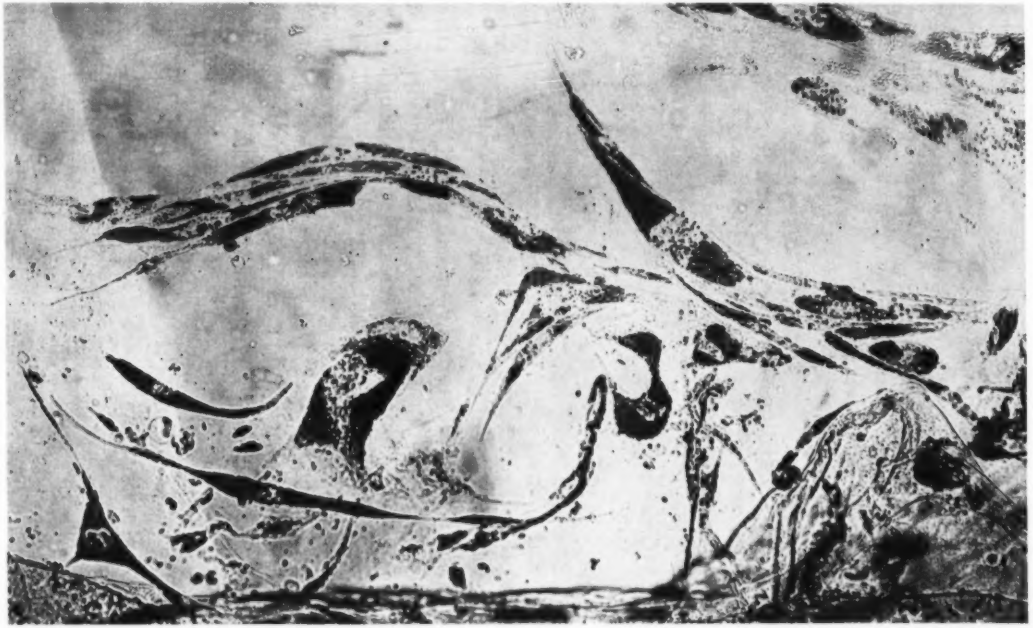

FIGURE 2

Eighth generation unstained culture showing pleomorphism, variations in granulation, and large nuclear clearings partially overlaid with granules. Fixed in 10% formalin. × 130

ing a factor specific for the maintenance of mast cells, it was discontinued.

Medium 5 (Leukemic Serum) offered little stimulation to migration or proliferation. The cells grew in an epithelial-like sheet, with very scant and fine granulation. Although the cells remained viable and some activity was noted there was insufficient growth for subculturing, even at the end of two months.

Media 8 and 9 (Cord Serum without EE_{50}) was placed on cultures with rounded and non-granulated cells. Although the spindled form and granulation were noted in some cultures within four days, it was doubtful that this response was significant, for it was subsequently observed that the cells became clear and rounded during a possible lag period in growth.

Medium 15 (Growth Hormone) appeared to be toxic at the level used. Growth after one week was scant; the cells were very small, granulated and vacuolated. At two weeks supravital staining with toluidine blue showed good evidence of metachromasia. These cultures after fixation and staining with Hematoxylin and Scharlach R gave evidence of fat droplets throughout the cytoplasm. This was the only time that fat was observed in the mast cells.

The fourth phase extended over a five-month period. It was characterized by the formation of granular and globular debris beneath the clots, and the gradual death of the cultures. Rapid subculturing and daily replacement of the medium were unsuccessful. Washes with trypsin offered a brief stimulation. Various new media were introduced in the hope of restoring the cultures to a more active state, and in an effort to curb the production of the granular and globular deposits.

Media 7, 10, 11 (Simms SUF) inhibited proliferation and caused marked differentiation within six days. The large, migrating cells were packed with coarse cytoplasmic granules, the nuclei were gross and contained a single nucleolus. At three weeks the cells became rounded and degenerate. No new cells were observed, no further migration occurred, dead cells were seen floating in the medium, and many cells were noted with ruptured membranes and released granules surrounding them.

Media 12 and 13 (Cord Serum and Simms SUF) maintained the cells, stimulated granulation and allowed proliferation at prolonged intervals. The nuclei were large and often contained two or more

FIGURE 3
Unstained culture showing the effect of serum ultrafiltrate. × 180

nucleoli. Mitotic division and some vacuolation were seen but the cells gave no evidence of fat when stained with Scharlach R.

The granular and globular debris appeared at the beginning of this phase. It occurred in the most actively growing cultures, during the second week following subculturing. Small dots surrounded the explant and increased in number and size until they formed a halo separating the growth zone completely from the explant. At times the deposits spread to the peripheral cells. Although the cells were not destroyed, they did not proliferate, and many became rounded.

Toward the end of this period the morphology changed. The cells became increasingly smaller and rounder, until they appeared as tiny refractile spheres. Where the spindle shape was retained the cells grew in twisted disorganized stalks. The peripheral cells appeared as mature forms, blunt and club-shaped, with thickened walls and large gaping nuclei. Supravital staining with toluidine blue demonstrated metachromasia in the cultures.

FIGURE 4
Mitotic spindle-shaped cell with second, smaller nucleus and two nucleoli. Stained with toluidine blue. × 720

Discussion

Three culturing techniques were tried repeatedly during the various phases of the experiment. Growth occurred only in Carrel flask cultures. The mast cell may require the semi-anaerobic condition supplied by a thick clot, or the oxygen tension afforded in the Carrel flask to promote proliferation.

The basic supernatant, Medium 6 (Cord Serum) provided an adequate growth stimulus for the mast cell during the major portion of the study. The serum-extract ratio of 10:1 protected the cells from excessive calcium phosphate precipitation. Media 12 and 13 (Cord Serum and Simms SUF) stimulated proliferation and granulation in the cells. It was unfortunate that these two media were used during

the last phase of the experiment when the cultures were no longer robust. It is possible that they might have provided a satisfactory source of nutrients had they been employed at an earlier time.

No clot lysis was noted in the cultures at any time. Many cell colonies were not transplanted for several months, and although the clots became opaque, excellent growth was stimulated through routine replacement of the medium. The absence of fibrinolysis in these cultures may be related to their ability to remain healthy in spite of extensive calcification.

Mitotic and amitotic divisions were noted in the study confirming the findings of Paff et al. (12). Although both forms were observed during the earlier phases of the experiment, only mitosis was seen in the terminal period. Since division in normal mast cells is reported by Michels (10) to be amitotic, the occurrence of mitotic forms in tissue cultures may be due to a struggle for adaptation to an alien environment.

Cytoplasmic granulation was observed to be cyclic. The number and size of the granules increased with the maturing process of the cells. No single cell, in an actively growing culture, could be followed through the cycle of development or granule formation, and our observations were drawn from the tissue culture as a composite study. In very active cultures, the granules were predominantly fine in contrast to the coarse granules in aging, migrating or dying cells of degenerate cultures. However, many cells were observed with both fine and coarse granules, and with areas free of any granulation. Staining with toluidine blue supravitally or in fixed preparations, demonstrated a corresponding variation in metachromasia. In some cells all the cytoplasmic granules stained, in others only a few granules could be stained, and in some cells only the long filaments showed the purple color.

No evidence of fat was demonstrated when the cells were stained with Scharlach R. Drennan (2) has reported a similar observation. Paff et al. (13) have revealed lipid granules with Sudan black B, a procedure not followed in this study. The occurrence of fat droplets in cultures maintained on Medium 15 (Growth Hormone) was not considered significant since the preparation appeared toxic at the level used.

It was not determined what caused the granular and globular debris which appeared during the final phase of the experiment, nor of what

it was composed. It may have been a cellular by-product or necrotic material from the explant, a manifestation of death in the cultures.

Only mast cells grew from the tissue fragments. This phenomenon was observed by Paff et al. (12) in their cultivation of mast cell tumors from the dog. The postulate of these investigators, that heparin was produced in the cultivated mast cells and acted to prevent the growth of other cells, is consistent with the findings of this study.

Summary

Human tissue mast cells from a biopsy of an infant with urticaria pigmentosa have been cultivated *in vitro* for thirteen months.

The tissue cultures underwent eight successful transfers in this period of time.

The cells stained metachromatically with toluidine blue, supravitally and in fixed preparations, thus fulfilling the accepted criterion for mast cells.

The cytoplasmic granules varied in size, number, and staining ability. The production and loss of granulation was cyclic.

The cells were observed to divide mitotically and amitotically.

No evidence of fat was demonstrated when the cells were stained with Scharlach R.

No clot lysis was noted in the tissue cultures.

Only mast cells grew from the original tissue fragments.

References

1. Bloom, F. 1942. *Arch. of Path.*, **33**, 661.
2. Drennan, J. M. 1951. *J. Path. and Bact.*, **63**, No. 3, 513-520.
3. Ehrich, W. E., Seifter, J., Alburn, H. E., & Begany, A. J. 1949. *Proc. Soc. Exp. Biol. and Med.*, **70**, 183-184.
4. Ellis, J. M. 1949. *Arch. Path.*, **48**, 426-435.
5. Friberg, U., Graf, W., & Aberg, B. 1951. *Acta Path. Microbiol. Scand.*, **29**, 197-202.
6. Gaillard, P. J. 1935. *Protoplasma*, **23**, 145-174.
7. Hanks, J. 1949. *Proc. Soc. Exp. Biol. Med.*, **71**, 328-334.
8. Holmgren, H., & Wilander, O. Z. 1937. *Mikr. Anat. Forsch.*, **42**, 242-278.
9. Jorpes, J. E. Heparin. 1946. Oxford Univ. Press.
10. Michels, N., Handbook of Hematology. 1938. Downey, N., Hoeber Inc., New York, 231-372.
11. Oliver, J. E., Bloom, F., & Mangieri, C. 1947. *J. Exp. Med.*, **86**, 107-116.
12. Paff, G. H., Bloom, F., & Reilly, C. 1947. *J. Exp. Med.*, **86**, 117-124.
13. ———, Montagna, W., & Bloom, F. 1947. *Cancer Res.*, **7**, 798-801.
14. ———, & Bloom, F. 1949. *Anat. Rec.*, **104**, 45.
15. Signorotti, B., Hull, W., & Kirk, P. L. 1950. *J. Gen. Physiol.*, **33**, 315-325.
16. Simms, H. S., & Stillman, N. P. 1937. *J. Gen. Physiol.*, **20**, 649.
17. Sutton, R. L., & Sutton, R. L. Jr. Diseases of the Skin, St. Louis, 1939.

Growth, 1953, **17**, 123-126.

RELATIONSHIP BETWEEN BODY WEIGHT AND GASTROCNEMIUS-SOLEUS MUSCLE WEIGHT IN GROWING RATS[1]

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The rat gastrocnemius-soleus muscle pair is frequently used in determining the effects of hormones, denervation and nutrition on the weight, growth and function of skeletal muscle. The weight of this muscle pair over a range of body weights should be known in order to (1) evaluate the effect of treatment on muscle weight and growth when the body weight is also altered by the treatment, and (2) predict muscle weight from body weight in studies where muscles of particular sizes are desired.

Invariably, gastrocnemius muscle weight is "corrected" for differences in body weight by expressing muscle weight as a simple fraction of body weight. The assumption which underlies this "correction" is that muscle weight is *directly* proportional to body weight. This is apparently true for the anterior tibialis muscle (1, 4) but is not true for the total body musculature (2).

The only data in the literature on the weights of gastrocnemius-soleus muscles from rats of widely different body weights were presented by Scow and coworkers (5). They found that this muscle pair is 0.35% of body weight in young rats weighing 37 grams (average) and 0.53% of body weight in older rats weighing 217 grams (average). These data imply that the weight of this muscle pair is not directly proportional to body weight and that a comparison of the ratio of gastrocnemius-soleus muscle weight to body weight, between experimental groups of different body weights, would be subject to systematic error.

In view of the foregoing and as a preliminary to studies on the effects of thyroid hormone on muscle weight and function, the relationship

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between gastrocnemius-soleus muscle weight and body weight in normal rats was investigated.

Materials and Methods

Ninety-five male Sprague-Dawley rats, 21 to 110 days old, weighing from 30 grams to 420 grams were used. Each rat was weighed and anesthetized with either Nembutal (5 mg./100 g.) or Barbital (35 mg./100 g.), the gastrocnemius-soleus muscle from either the right or left side was dissected free, and the tendon of Achilles clipped close to the muscle. The muscle pair was weighed to the nearest 0.01 gram, dried for twenty-four hours at 100° C., and reweighed.

The muscles were removed bilaterally (alternating right and left side as the first muscle dissected) from fifty-two of the animals to check the effect of order of dissection and dissection technique on muscle weight. There was no significant difference between the right and left muscles with respect to either wet or dry weights. The wet weight of the *first* muscle dissected was slightly but significantly greater than that of the second muscle; the dry weights again not differing significantly. The muscle weights reported below are for the first muscle removed from each animal.

Results

When the gastrocnemius-soleus muscle weights (wet) are plotted as a function of body weight (Figure 1) a curvilinear relationship is revealed. This curve is rectified by plotting the logarithms of both variables, indicating that the relationship between muscle weight and body weight can be described by the general equation, Muscle Weight $= A\ \text{Body Weight}^B$. When the muscle weight is expressed in milligrams, and the body weight in grams, the constants calculated from the data are $A = 1.77$, $B = 1.24$ ($+$ sy $\cdot$ x $= 7.6\%$).

Over the weight range studied, the dry weight of the muscle appears linearly related to the wet weight. The calculated regression line, when both weights are expressed in grams, is Dry Weight $= -0.012 + 0.242$ Wet Weight. The standard deviation from regression is $\pm$ .0095, the standard error of the slope is $\pm$ .0013, and the standard error of the intercept is $\pm$.0019. The significant deviation of the intercept ($-$.012) from the origin is probably a reflection of the greater percentage of muscle water in fetal and preweaning rats (3), and it indicates that the relationship between wet and dry weights departs from linearity at very low muscle weights.

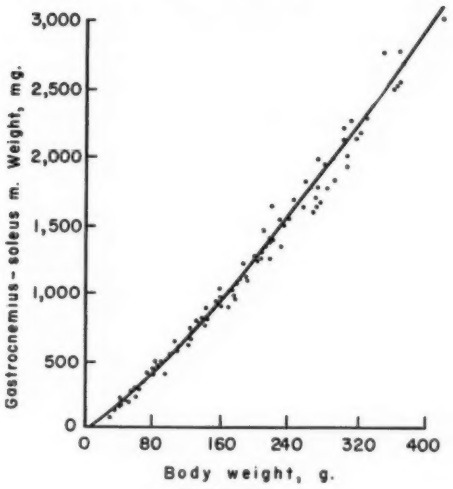

FIGURE 1

The relationship between body weight and gastrocnemius-soleus muscle (wet) weight in growing male rats. The curve is drawn according to the equation calculated from the data, Muscle Wt., mg. = 1.77 Body Wt. $^{1.24}$, g. Each dot represents the weight of the first gastrocnemius-soleus muscle removed from the rat.

Discussion

As can be seen from Figure 1, the ratio of gastrocnemius muscle weight to body weight is not constant throughout a wide range of body weights. For these data,

$$\frac{\text{Muscle Wt., mg.}}{\text{Body Wt., g.}} = 5.96 \pm .96 \text{ (Coeff. of var.} = 16.1\%),$$

whereas $\dfrac{\text{Muscle Wt., mg.}}{\text{Body Wt.}^{1.24}\text{, g.}} = 1.76 \pm .13$ (Coeff. of Var. $= 7.3\%$).

The apparent reduction in random variation which results from using body weight raised to the 1.24 power as the denominator is a reflection of the constancy of this ratio throughout the total range of body weights. On the other hand, the simple ratio of muscle weight to body weight changes systematically from around 4 to 7.5 with increasing body weights, rendering this calculation worthless as a basis for comparison of muscle weights in animals of different body weights.

It should be noted that the aforementioned data of Scow and co-

workers (5) yield a value for $\frac{\text{Muscle Wt., mg.}}{\text{Body Wt.}^{1.24}, \text{g.}}$ of 1.47 for the 37 gram rats, and 1.45 for the 217 gram rats. The lower value for the constant may be due to the fact that the animals were dead before the dissection, since we have found that the constant for rats killed before the muscle was removed was equal to $1.48 \pm .099$ ($n = 7$).

Summary

In growing male Sprague-Dawley rats, the following relationship was found between body weight and the weight of the gastrocnemius-soleus muscle pair, Muscle wt., mg. = 1.77 Body wt.$^{1.24}$, g. Because of the curvilinearity of the relationship, the ratio $\frac{\text{Muscle wt.}}{\text{Body wt.}}$ changes as the body weight changes, and therefore this ratio should not be used in comparing muscle weights in rats of different body weights. The ratio $\frac{\text{Muscle wt.}}{\text{Body wt.}^{1.24}}$ is constant throughout the body weight range tested (30 to 420 grams).

References

1. Cameron, A. T. 1925. Normal variations of percentage weights of body organs of the albino rat with changing body weight. *Am. J. of Physiol.*, **74**, 151-157.
2. Jackson, C. M., & Lowry, L. G. 1912. On the relative growth of the component parts (head, trunk and extremities) and systems (skin, skeleton, musculature and viscera) of the albino rat. *Anat. Rec.*, **6**, 449-474.
3. Lowry, L. G. 1913. The growth of the dry substance in the albino rat. *Anat. Rec.*, **7**, 143-168.
4. Moment, G. B. 1933. The effects of rate of growth on the post-natal development of the white rat. *J. Exp. Zool.*, **65**, 359-393.
5. Scow, R. O., *et al.* 1949. Response by the rat thyro-parathyroidectomized at birth to growth hormone and to thyroxin given separately or in combination. *Anat. Rec.*, **104**, 445-463.

Growth, 1953, **17**, 127-128.

BOOK REVIEW

Genetic—Statistical and Psychiatric Investigations of a West Swedish Population, by Torsten Sjögren. Acta Psychiatrica et Neurologica, Supplementum 52. Ejnar Munksgaard, Copenhagen, 1948. 102 pp.

Morbus Alzheimer and Morbus Pick, A Genetic, Clinical and Patho-Anatomical Study, by Torsten Sjögren, Hakon Sjögren, and Åke G. H. Lindgren. Acta Psychiatrica et Neurologica, Supplementum 82. Ejnar Munksgaard, Copenhagen, 1952. 152 pp.

Reviewed by Earl L. Green

These two studies are characterized by meticulous attention to details and thorough reporting of results of analyses of familial data on psychiatric and medical variations of genetic interest.

The first deals with the occurrence of psychoses and oligophrenia over a long period of time in the human populations of two islands on the west coast of Sweden. Information was obtained from the parish registers of the islands for the years 1900-1944 and from the pertinent hospital case-histories for the same period. The probands, of which there were 335, comprised 255 psychotics, 9 psychopathics and 71 oligophrenics. Affected siblings of the probands, of which there were 62, comprised 22 psychotics, 22 psychopathics, and 18 oligophrenics. 6700 ancestors were investigated by means of the parish registers. The data available which are reproduced as completely as possible in tables, appendices, and a pedigree chart, permit the estimation of consanguinity of the parents, the calculation of the morbidity risks, and the expectation of psychoses among the siblings of the probands.

The second study is in three parts dealing separately with the genetic, clinical, and pathological aspects of Morbus Alzheimer and Morbus Pick, which are both types of presenile cerebral atrophy. Information was obtained from the case records of certain mental hospitals and of certain university institutions in Sweden. There were 18 probands with diagnosis of Morbus Alzheimer, 18 with diagnosis Morbus Pick, and 44 with a clinical diagnosis of Pick-Alzheimer syndrome. It is found that Morbus Pick may depend upon a dominant

major gene with modifiers and that Morbus Alzheimer may depend upon several pairs of quantitative genes. The data are presented in extensive tables and figures.

Growth and Culture: A Photographic Study of Balinese Childhood. Margaret Mead and Frances Cooke Macgregor. 1951. (223 pp., $7.50, G. P. Putnam's Sons, New York).

This is a detailed account of maturational patterns of Balinese infants and young children, told through the analysis of a large series of photographs taken in Bali, 1936-38. The text is illustrated with some 400 photographs of representative children in a wide variety of activities.

Observations have been related to the Gesell standards, and the same general patterns are identified, but in addition certain Balinese peculiarities, and areas of increased emphasis and contrast, are described and illustrated. It was hoped to interpret the Balinese data in terms of the Gesell-Ilg scheme of spiral development, but, as Mead remarks, the findings did not contribute heavily in this respect.

As an appendix, there is a discussion of field techniques, and of the assumptions made and procedures used. As an insight into the problems and approaches of the field worker, this section is particularly informative.

Finally, *Growth and Culture* contains, in its opening two chapters, certain cogent remarks about research in child development in the United States, and about its use and abuse by parents and lay-groups. These two chapters are, in the writer's opinion, the best part of the book, and should be required reading for all who are concerned, whether practitioner or theoretician, with problems of child development.

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Atomic Bomb Casualty Commission
Hiroshima